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Biochemical and Biophysical Research Communications 331 (2005) 23–30
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Identification of Zfp-57 as a downstream molecule of STAT3 and Oct-3/4 in embryonic stem cells

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Received 7 March 2005
Available online 25 March 2005

Abstract

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocysts. Transcription factor STAT3 is essential for the self-renewal of ES cells. In this study, we searched for downstream molecules of STAT3 in ES cells. Using DNA chip analysis, we obtained zinc finger protein (Zfp)-57. The expression of Zfp-57 was restricted to undifferentiated ES cells and activation of STAT3 led to expression of Zfp-57. We also found that forced expression of a dominant-negative mutant of STAT3 or repression of Oct-3/4 expression led to down-regulation of Zfp-57. Targeted disruption of Zfp-57 resulted in no gross phenotypical defects, including expression of undifferentiated-state-specific genes. These data suggest that Zfp-57 is a downstream molecule of STAT3 and Oct-3/4 in ES cells, although dispensable for their self-renewal.

Keywords: ES cells; STAT3; Oct-3/4; Zfp-57; Self-renewal; Targeted disruption; RNAi

Mouse embryonic stem (ES) cells derived from the inner cell mass of blastocysts possess two major abilities: differentiation into many types of cells, pluripotency; and production of two identical stem-cell daughters upon cell division, self-renewal [1,2].

The pluripotency and self-renewal of mouse ES cells can be maintained in the presence of leukemia inhibitory factor (LIF) [3,4]. LIF stimulation leads to activation of transcription factor STAT3, which plays a major role in the self-renewal of ES cells [5,6]. Previously, we generated ES cells expressing STAT3ER, a fusion protein consisting of STAT3 and the ligand-binding domain of estrogen receptor, and found that their pluripotency can be maintained by stimulation with a synthetic estrogen receptor ligand, 4-hydroxytamoxifen (4HT), even in the absence of LIF [5]. Another study showed that forced expression of a dominant-negative mutant of STAT3 caused differentiation of ES cells in the presence of LIF [6]. In addition to STAT3, POU transcription factor Oct-3/4 is also required for the self-renewal of ES cells. Oct-3/4 is expressed specifically in pluripotent cells such as ES, early embryo, and germ cells [7,8]. Targeted disruption of the Oct-3/4 gene results in loss of the pluripotent inner cell mass, and conditional repression of this gene in ES cells leads to differentiation into trophoectoderm, demonstrating that Oct-3/4 plays an essential role in the self-renewal of ES cells [9,10]. Forced expression of Oct-3/4, however, cannot maintain the self-renewal of ES cells in the absence of LIF [10].

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.03.118

Thus, the activation of STAT3, but not the expression of Oct-3/4, is sufficient for the self-renewal of ES cells. These findings prompted us to search for a downstream target gene of STAT3 in ES cells.

Here we performed DNA chip analysis and obtained zinc finger protein (Zfp)-57. Zfp-57 was originally cloned from a mouse teratocarcinoma stem cell line, F9, as an undifferentiated-cell-specific gene, using the promoter trap technique [11]. The expression of Zfp-57 is restricted to testis and brain. The protein, containing five repeats of the zinc finger motif and the KARB domain, is known to be localized in the nucleus in F9 and Schwann cells. It was also suggested that Zfp-57 acts as a transcriptional repressor in Schwann cells [12]. Little is known, however, about the expression and function of Zfp-57 in ES cells. In the present study, we examined the expression of Zfp-57 in ES cells and performed gene disruption to study its role in their self-renewal.

Materials and methods

Cell culture. ES cell lines, A3-1 derived from embryos of 129/SvJ inbred strain [13] and ZHBTc4 [10], were cultured on gelatin-coated dishes with Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) containing 15% fetal bovine serum, 2 mM L-glutamine, 1× non-essential amino acid (Cell and Molecular Technologies, Phillipsburg, NJ, USA), 1× nucleosides mix (Cell and Molecular Technologies), 40 μM β-mercaptoethanol (Sigma), and 0.1% mouse LIF, produced as conditioned media from human embryonic kidney 293 cells expressing LIF from a transiently transfected plasmid. STAT3ER ES cells were cultured in the same media except that it contained 1 μM 4-hydroxytamoxifen (4HT, Sigma) instead of LIF [5].

Plasmid construction and transfection. The mammalian expression vector pCAG-IP was constructed by inserting the sequence of internal ribosomal entry site (IRES) and the puromycin-resistance gene into the plasmid pCAGGS [14,15]. Plasmids pCAG-wtSTAT3-IP and pCAG-dnSTAT3-IP were constructed by inserting cDNA for wild-type STAT3 (wtSTAT3) and a dominant-negative mutant of STAT3 (dnSTAT3) into pCAG-IP, respectively. pSilencer 1.0-U6 (Ambion, Austin, TX, USA) was used as an RNAi expression vector. To generate pSi-puro, a puromycin-resistance gene of pPUR (Clontech, Palo Alto, CA, USA) was introduced into a non-coding region of the pSilencer 1.0-U6. A target sequence for RNA interference (RNAi) of Zfp-57 (5′-CCG TCA CCT GAA GGT ACA C-3′) was determined by B-Bridge International (Sunnyvale, CA, USA). The sense and antisense oligonucleotides were annealed in a buffer [100 mM potassium-acetate, 30 mM Hepes–KOH (pH 7.4), and 2 mM magnesium-acetate], and cloned into the *Apa*I and *Eco*RI sites of pSi-puro to produce a Zfp-57 RNAi expression vector (pSi-puro-Zfp-57). The construct was sequenced before transfection. ES cells (4×10^5 cells) in a 6-cm dish were transfected with pCAG-IP, pSi-puro or their derivatives using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA). Two days after transfection, ES cells were treated with 1 μg/ml puromycin (Nacalai Tesque, Kyoto, Japan) for several days and then subjected to RT-PCR, Northern or Western blot analyses.

Preparation of antibody and Western blot analysis. Anti-Zfp-57 antibody was generated with a synthetic peptide (NPQSRKGKGY KAQHRGERPC) [11] by Operon Biotechnologies (Alameda, CA, USA). ES cells were harvested and lysed in a cell lysis buffer [20 mM Hepes–NaOH (pH 7.2), 10 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium fluoride, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1% Triton X-100, and 10% glycerol]. The cell lysates were mixed with 5× SDS-sample buffer [50 mM Tris–HCl (pH 6.8), 30% glycerol, 10% SDS, 250 mM dithiothreitol, 10 mM EDTA, and 0.01% Coomassie brilliant blue R250], subjected to SDS–10% polyacrylamide gel electrophoresis (PAGE), and electroblotted onto a nitrocellulose membrane. The membrane was incubated with rabbit anti-Zfp-57 antibody, followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Upstate Biotechnology, Charlottesville, VA, USA). Signals were detected with LAS-1000 (Fuji Film, Tokyo, Japan). The membrane was then reprobed with monoclonal anti-α-tubulin antibody (ICN Biomedical, Irvine, CA, USA), followed by HRP-conjugated goat anti-mouse IgG (Upstate Biotechnology). Reproducibility of these analyses was confirmed by several independent experiments.

Northern blot and RT-PCR analyses. Total RNAs were isolated from ES cells with Trizol reagent (Invitrogen). Total RNA (10 μg) was separated by electrophoresis in denaturing 1.0% agarose gel containing 2.2 M formaldehyde, and transferred to Hybond-N+ nylon membrane (Amersham Biosciences, Piscataway, NJ, USA), followed by UV cross-linking [16]. The membrane was hybridized with α-^{32}P-labeled probe of full-length Zfp-57, Rex-1, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA in QuikHyb (Stratagene, La Jolla, CA, USA) at 68 °C for 2 h. The membranes were washed in 0.2× SSC, 0.1% SDS at 68 °C, and radiolabeled bands were visualized with an image analyzer (BAS-2000, Fuji Film).

For RT-PCR analysis, 1 μg of total RNA was converted to cDNA by Superscript II reverse transcriptase (Invitrogen) with oligo(dT)$_{12–18}$ primers (Amersham Biosciences). PCR was performed with the following primers: 5′-TGT AAT TTC TGT GGC AAG AC-3′ and 5′-TGT GGA TCT TGA GGT GGG TTA A-3′ for Zfp-57, 5′-CGC CCT GAG GAA GCA-3′ and 5′-CCT TCA GCA TTT CTT CCC TGC CTT TGC-3′ for Rex-1, 5′-GCC CTG CAG AAG GAG CTA GAA C-3′ and 5′-GGA ATA CTC AAT ACT TGA TCT-3′ for Oct-3/4, 5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′ and 5′-TCC TTG GAG GCC ATG TAG GCC AT-3′ for GAPDH, and 5′-ATG GGC TCT GTG GTC AAG TC-3′, and 5′-ACC CCT CCC AAT TCT CTT GT-3′ for Sox-2. Primers for hypoxanthine guanine phosphoribosyltransferase (HPRT), Nanog, Gata4, Fgf5, and Brachyury T were described before [10,17]. Reproducibility of these analyses was confirmed by several independent experiments.

Construction of targeting vectors and electroporation. The 2.5-kb 5′ short arm (including exon 2 (−1493 to +938) of the Zfp-57 gene) and the 4.5-kb 3′ long arm (including 3′ flanking region (+5738 to +10312) of the Zfp-57 gene) were isolated by PCR and verified by partial sequencing. The translation start site was position +1. The short and long arms were subcloned into the *Kpn*I and *Not*I sites of pBluescript II KS (−) vector, respectively. Drug-resistance cassette vectors, pGT 1.8 IRES-hygro-pA2 [18] and pGT1.8 IRES-neo-pA, were digested with *Sal*I, and the fragment containing a drug-resistance gene was cloned into the *Sal*I site of the pBluescript II KS (−) vector carrying the short and long arms. Targeting vectors were linearized and electroporated into ES cells at 240 V, 500 μF. Cells were selected with 375 μg/ml G418 or 300 μg/ml hygromycin (Nacalai Tesque), picked up, and then analyzed by genomic Southern blot analysis.

Genomic Southern blot and PCR analyses. The 473-bp probe A and 456-bp probe B for Southern blot analysis were isolated by PCR. Primers used were as follows: 5′-GAA AGG CTC ACG TGG CTG A-3′ and 5′-TAC CTC CAT CCA CCT CCT CA-3′ for probe A, and 5′-ACC ACA TGG TTG GCT TGT TC-3′ and 5′-TTC TCC AAG TCT CCA AGT AGG G-3′ for probe B. The amplified fragments were cloned into pCR 2.1 vector (Invitrogen) and verified by sequencing. Genomic DNA of ES cells was isolated as described before [19], digested with *Bgl*I (for probe A) or *Bam*HI (for probe B), and subjected to 1.0% agarose gel electrophoresis. The gel was soaked in 0.25 M HCl, neutralized in 0.4 N NaOH, and then the separated genomic DNA was transferred to Hybond-N+ nylon membrane (Amersham Biosciences). After UV cross-linking [16], the membrane was hybridized with

α-^{32}P-labeled probe in QuikHyb at 68 °C for overnight. The membrane was washed in 0.2× SSC, 0.1% SDS at 68 °C, and radiolabeled bands were visualized with BAS-2000. In the case of genomic DNA PCR, neomycin-, and hygromycin-resistance genes were amplified by PCR with the genomic DNAs as template. Primers used were as follows: 5′-GAA GGG ACT GGC TGC TAT TG-3′ and 5′-AAT ATC ACG GGT AGC CAA CG-3′ for the neomycin-resistance gene, 5′-GAA TTC AGC GAG AGC CTG AC-3′ and 5′-GAT GTT GGC GAC CTC GTA TT-3′ for the hygromycin-resistance gene, and 5′-TGT AAT TTC TGT GGC AAG AC-3′ and 5′-TGT GGA TCT TGA GGT GGG TTA A-3′ for Zfp-57 exon 4.

Flow cytometric analysis. For 5-bromo-2′-deoxyuridine (BrdU, Nacalai Tesque) incorporation assay, ES cells were incubated with BrdU for 30 min, fixed in 70% ethanol at −20 °C for 2 h, incubated in 2 M HCl for 20 min, and neutralized with 0.1 M sodium borate (pH 8.5) for 2 min. The cells were stained with a monoclonal anti-BrdU antibody (NeoMarkers, Fermont, CA, USA), followed by fluorescein isothiocyanate (FITC)-conjugated bovine anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were counterstained with propidium iodide (PI, Nacalai Tesque). Flow cytometric analysis was performed on Desktop Cell Sorter JSAN (Bay Bioscience, Kobe, Japan).

Results

Expression of Zfp-57 mRNAs and protein is restricted to undifferentiated ES cells

First, we searched for a downstream molecule of STAT3 using STAT3ER-expressing ES cells (STAT3ER ES cells). DNA chip analysis [20] revealed that zinc finger protein (Zfp)-57 showed a higher expression level in undifferentiated STAT3ER ES cells than differentiated cells (data not shown). To confirm this, we performed Northern and Western blot analyses (Fig. 1). As reported previously in F9 cells [11], two transcripts of Zfp-57 mRNAs, which differ in the sequence of the 3′-untranslated region, were detected in ES cells cultured with LIF (Fig. 1A). When ES cells were allowed to differentiate by removal of LIF, the signals of the two transcripts were reduced. Differentiation of ES cells was confirmed by the decreased expression of Rex-1, a self-renewal marker. Western blot analysis also showed that expression of Zfp-57 protein (about 50 kDa) was down-regulated after withdrawal of LIF for 5 days (Fig. 1B). These results indicated that the expression of Zfp-57 is restricted to undifferentiated ES cells and depends on LIF stimulation.

Zfp-57 expression is regulated by both STAT3 and Oct-3/4

It is well known that LIF stimulation directly leads to activation of STAT3 in ES cells, and that Oct-3/4 is expressed in ES cells cultured with LIF. We therefore investigated whether Zfp-57 is a downstream molecule of STAT3 and/or Oct-3/4.

STAT3ER ES cells were cultured in the presence or absence of 4HT for 4 or 6 days, and expression of Zfp-57 mRNA was examined by RT-PCR analysis

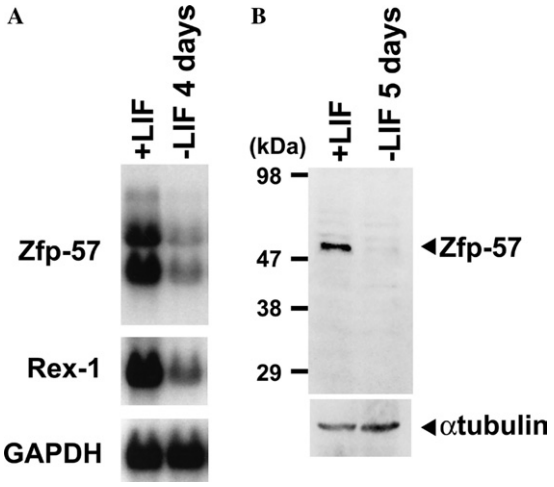

Fig. 1. Expression of Zfp-57 mRNA and protein is restricted to undifferentiated ES cells. (A) Northern blot analysis. After 4-day culture with or without LIF, ES cells were harvested and total RNAs were isolated. Samples containing 10 μg of total RNAs were separated by 1.0% agarose gel. GAPDH was used as an internal control. (B) Western blot analysis. ES cells cultured with or without LIF for 5 days were harvested and lysed in cell lysis buffer. Samples containing 50 μg of protein were subjected to immunoblotting with anti-Zfp-57 antibody (upper panel) or anti-α-tubulin monoclonal antibody (lower panel). α-Tubulin was used as an internal control.

(Fig. 2A). The band of Zfp-57 was strongly detected in STAT3ER ES cells cultured with 4HT, and its signal decreased after removal of 4HT. Western blot analysis also showed that the expression of Zfp-57 protein was restricted to 4HT-stimulated STAT3ER ES cells (Fig. 2B). Next we examined the effect of a dominant-negative mutant of STAT3 (dnSTAT3) on Zfp-57 expression. As for dnSTAT3, we used STAT3[Y705F], which was reported to block the activation of endogenous STAT3 [21]. ES cells were transfected with the empty, wild-type STAT3 (wtSTAT3) or dnSTAT3 expression vector. Three days after puromycin treatment, the expression of Zfp-57 mRNA was determined by RT-PCR analysis (Fig. 3A). The band of Zfp-57 was detected in ES cells transfected with the empty or wtSTAT3 expression vector, whereas its signal was strongly reduced in ES cells expressing dnSTAT3. Taken together, these results indicated that Zfp-57 is a downstream target of STAT3 in ES cells.

Next we examined whether Zfp-57 is a downstream molecule of Oct-3/4 utilizing ZHBTc4 ES cells, in which tetracycline (Tet) stimulation leads to down-regulation of Oct-3/4 expression [10]. ZHBTc4 ES cells were treated with or without Tet for 24 or 48 h, and the expression of Zfp-57 and Oct-3/4 mRNAs was determined by RT-PCR analysis. As shown in Fig. 3B, Tet stimulation for 24 h completely repressed the expression of Oct-3/4 in ZHBTc4 ES cells, which was recovered by removal of Tet. Similarly, the expression of Zfp-57 mRNA was decreased with Tet stimulation and

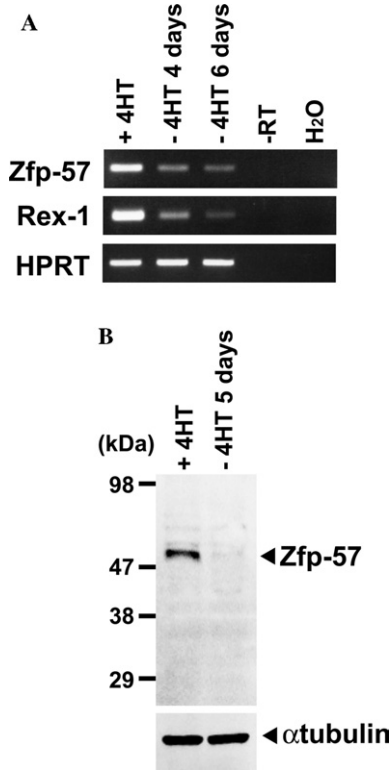

Fig. 2. Expression of Zfp-57 mRNA and protein can be maintained by STAT3ER. (A) RT-PCR analysis. STAT3ER ES cells were cultured with or without 4HT for 4 or 6 days. Total RNAs were isolated and subjected to RT-PCR analysis. PCR products of Zfp-57, Rex-1, and HPRT were amplified using specific primers and subjected to 1.5% agarose gel. HPRT was used as an internal control. (B) Western blot analysis. After 5-day culture with or without 4HT, STAT3ER ES cells were harvested and lysed in cell lysis buffer. Signals of Zfp-57 (upper panel) and α-tubulin (lower panel) were detected by immunoblotting with anti-Zfp-57 and anti-α-tubulin antibodies, respectively.

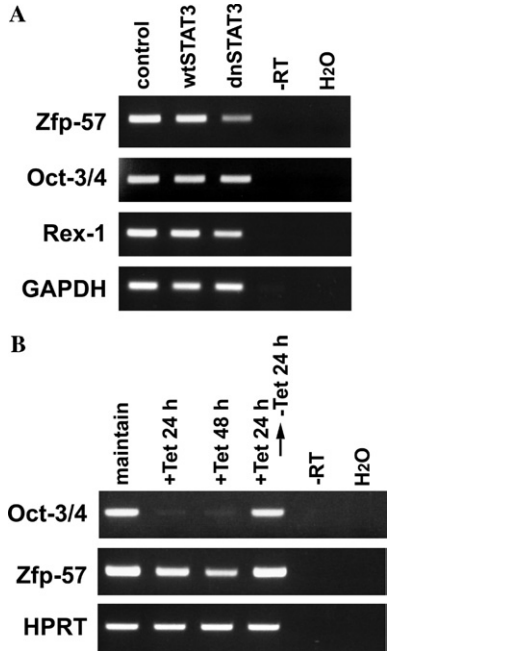

Fig. 3. Zfp-57 expression is regulated by both STAT3 and Oct-3/4. (A) Effect of a dominant-negative mutant of STAT3 (dnSTAT3) on Zfp-57 expression. ES cells transfected with the empty, wild-type STAT3 (wtSTAT3), and dnSTAT3 expression vectors were harvested, and total RNAs were isolated. RT-PCR analysis was performed using specific primers for Zfp-57, Oct-3/4, Rex-1, and GAPDH. (B) Role of Oct-3/4 in Zfp-57 expression. ZHBTc4 ES cells treated with or without Tet for the indicated period were harvested, and total RNAs isolated from these cells were subjected to RT-PCR analysis using specific primers for Oct-3/4, Zfp-57, and HPRT.

recovered after Tet removal. These results suggested that Oct-3/4 also controls the expression of Zfp-57 in ES cells.

Zfp-57 RNAi-expressing ES cells maintain an undifferentiated state

The results described above suggest the involvement of STAT3 and Oct-3/4 in the expression of Zfp-57. Since these two molecules play crucial roles in the self-renewal of ES cells, it was possible that Zfp-57 might be involved in their self-renewal. To explore this possibility, we tried to down-regulate Zfp-57 by RNA interference (RNAi). ES cells were transfected with the empty or Zfp-57 RNAi expression vector (pSi-puro-Zfp-57) and cultured for several days with puromycin. Among several drug-resistant clones, we picked one. As shown in Fig. 4A, the expression of Zfp-57 protein was drastically suppressed in this clone. The reduction in Zfp-57 mRNA was also confirmed by RT-PCR analysis (Fig. 4B). On the other hand, however, expression levels of

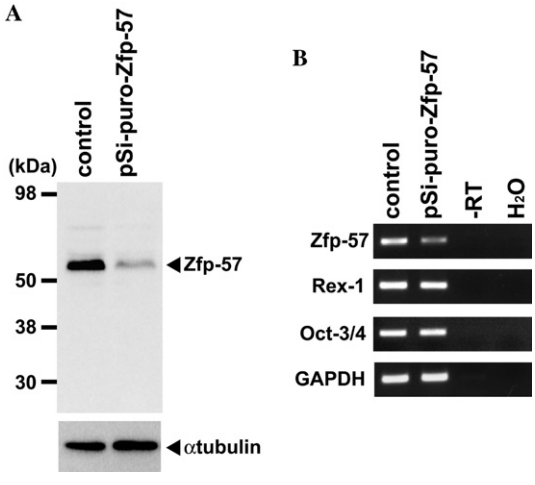

Fig. 4. Zfp-57 RNAi-expressing ES cells maintain an undifferentiated state. (A) Western blot analysis. Zfp-57 RNAi-expressing ES cells were harvested and lysed in cell lysis buffer. Samples containing 50 μg of protein were subjected to immunoblotting with anti-Zfp-57 antibody (upper panel) or anti-α-tubulin antibody (lower panel). (B) RT-PCR analysis. Total RNAs were isolated from Zfp-57 RNAi-expressing ES cells and subjected to RT-PCR analysis. PCR products of Zfp-57, Rex-1, Oct-3/4, and GAPDH were amplified using specific primers and subjected to 1.5% agarose gel.

undifferentiated-state-specific genes, Oct-3/4 and Rex-1, were maintained. These results suggested that Zfp-57 RNAi-expressing ES cells maintain the undifferentiated state, and raised the possibility that Zfp-57 might not be involved in the self-renewal of ES cells.

Targeted disruption of Zfp-57 in ES cells

Since the Zfp-57 RNAi did not repress the expression of Zfp-57 completely (Fig. 4), we generated Zfp-57-deficient ES cells using homologous recombination. We constructed a gene-targeting vector by which the entire coding region of Zfp-57, except for N-terminal six amino acids, can be replaced with neomycin (neo)- or hygromycin (hygro)-resistance gene (Fig. 5A). After electroporation of the linearized neomycin-resistance targeting vector into ES cells, we obtained two clones (1E11 and 1F9), out of 72 clones, as heterozygous Zfp-57 mutant ES cells (Zfp-57$^{+/-}$). Next, the wild-type allele of the Zfp-57$^{+/-}$ ES cells (1F9) was further disrupted with the hygromycin-resistance targeting vector, and two clones (1A12 and 2E9), out of 121 clones, were obtained as homozygous Zfp-57 mutant ES cells (Zfp-57$^{-/-}$). When we carried out genomic Southern blot analysis, the Zfp-57$^{+/-}$ ES cell clone, 1F9, showed a 3.2 kb *Bgl*I fragment (disrupted allele) in addition to a 5.2 kb *Bgl*I fragment (wild-type allele) with probe A, and a 5.0 kb *Bam*HI fragment (disrupted allele) besides a 8.7 kb *Bam*HI fragment (wild-type allele) with probe B (Fig. 5B). On the other hand, Zfp-57$^{-/-}$ ES cell clones, 1A12 and 2E9, showed a single band of a 3.2 kb *Bgl*I fragment (probe A) and a 5.0 kb *Bam*HI fragment (probe B). These ES cells were genotyped by genomic DNA PCR. A band of Zfp-57 exon 4 was amplified from genomic DNA of wild-type (WT) and Zfp-57$^{+/-}$ (1F9) ES cells, a band of the neomycin-resistance gene

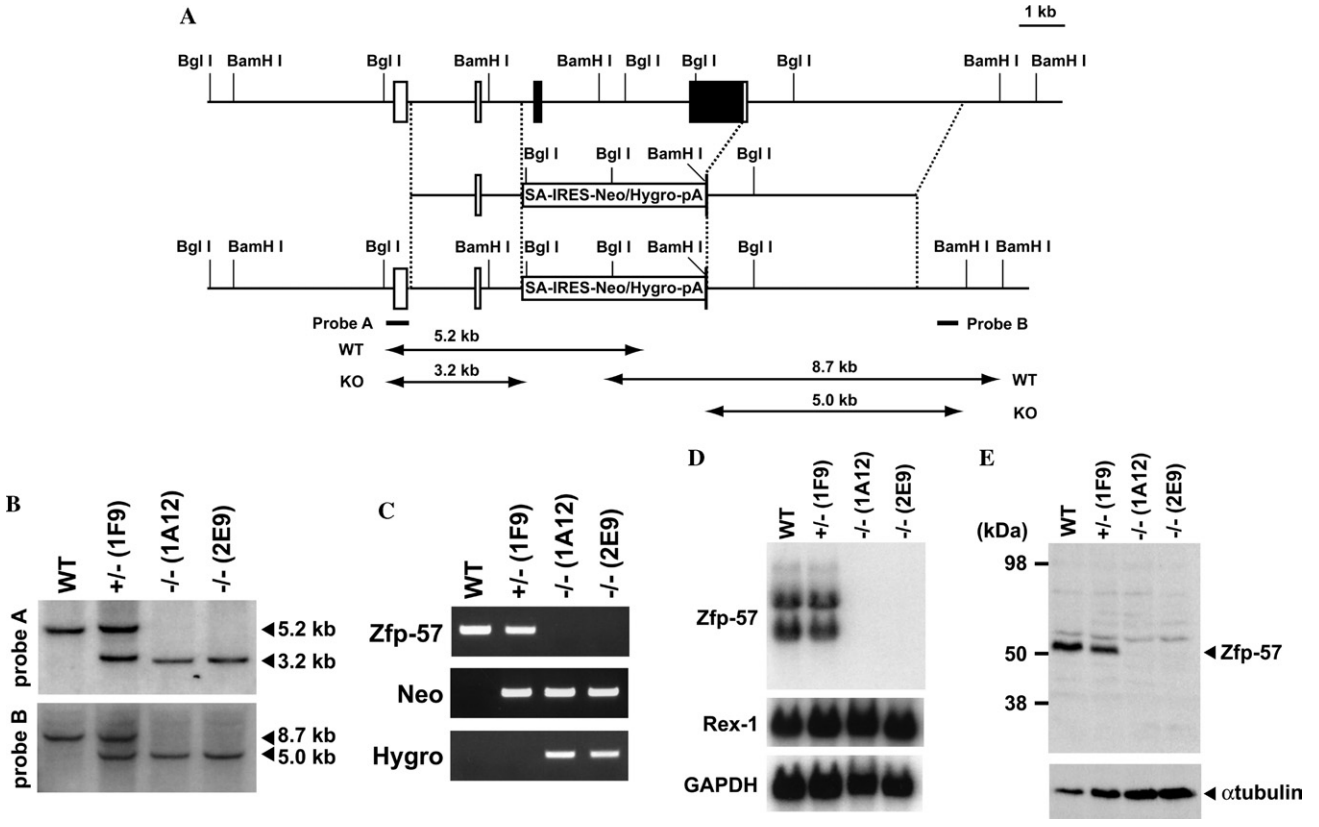

Fig. 5. Targeted disruption of the Zfp-57 gene. (A) Targeting strategy of Zfp-57 deletion. Thick lines indicate flanking regions and introns of Zfp-57 gene. Black boxes and white boxes indicate coding regions and untranslated regions of Zfp-57 gene, respectively. The entire coding region of Zfp-57 gene, except for N-terminal six amino acids, was replaced by neomycin (Neo)- or hygromycin (Hygro)-resistance gene, En-2 splice acceptor (SA), IRES, and poly(A) additional sequence (pA) [18]. Closed bars denote the locations of probes A and B used for Southern blot analyses. (B) Southern blot analysis. Genomic DNAs isolated from wild-type (WT), Zfp-57$^{+/-}$ (+/−), and Zfp-57$^{-/-}$ (−/−) ES cells were digested with *Bgl*I or *Bam*HI, separated by 1.0% agarose gel, transferred to Hybond-N+ membrane, and hybridized with α-^{32}P-labeled probe A or B, respectively. (C) Genomic DNA PCR analysis. Wild-type (WT), Zfp-57$^{+/-}$ (+/−), and Zfp-57$^{-/-}$ (−/−) ES cells were genotyped by genomic DNA PCR. PCR products of Zfp-57 exon 4, neomycin- and hygromycin-resistance genes were amplified using specific primers and subjected to 1.5% agarose gel. (D) Northern blot analysis. Total RNAs were isolated from ES cells and separated by 1.0% agarose gel. (E) Western blot analysis. ES cells were harvested and lysed in cell lysis buffer. Signals of Zfp-57 (upper panel) and α-tubulin (lower panel) were detected by immunoblotting with anti-Zfp-57 and anti-α-tubulin antibodies, respectively.

was from Zfp-57$^{+/-}$ and Zfp-57$^{-/-}$ (1A12 and 2E9) ES cells, and a band of the hygromycin-resistance gene was from Zfp-57$^{-/-}$ ES cells (Fig. 5C).

To examine the expression level of Zfp-57 in these ES cells, we performed Northern and Western blot analyses. As shown in Fig. 5D, two bands of Zfp-57 mRNAs were detected in WT and Zfp-57$^{+/-}$ ES cells. On the other hand, no signals were detected in Zfp-57$^{-/-}$ ES cells. Western blot analysis also revealed Zfp-57 is not expressed in Zfp-57$^{-/-}$ ES cells (Fig. 5E). These results clearly showed no expression of Zfp-57 mRNA or protein in the Zfp-57$^{-/-}$ ES cells.

Zfp-57 is dispensable for self-renewal of ES cells

When we compared morphology among wild-type, Zfp-57$^{+/-}$, and Zfp-57$^{-/-}$ ES cells, all formed compact colonies, suggesting that Zfp-57$^{-/-}$ ES cells are self-renewing (Fig. 6A). To confirm this, we examined expression of various marker genes for self-renewal, including Rex-1, Oct-3/4, Nanog, and Sox2 [10,17,22–24], and for ES differentiation, such as Gata4 (primitive endoderm marker), Brachyury T (mesoderm marker), and Fgf5 (embryonic ectoderm marker). Northern blot and RT-PCR analyses revealed that all the self-renewal markers were expressed in Zfp-57$^{-/-}$ ES cells, as well as WT and Zfp-57$^{+/-}$ ES cells (Figs. 5D and 6B), and the induction of Gata4, Fgf5, and Brachyury T was hardly detected (Fig. 6B). Finally, we examined the growth rate of these cells. BrdU incorporation assay showed that about 60% of ES cells were BrdU-positive and there were no significant differences among WT, Zfp-57$^{+/-}$, and Zfp-57$^{-/-}$ ES cells (Fig. 6C). Cell cycle distribution was also normal in Zfp-57$^{-/-}$ ES cells (data not shown), suggesting that Zfp-57 is not involved in proliferation of ES cells.

Taken together, these results indicated that Zfp-57 is dispensable for self-renewal of ES cells.

Discussion

In the present study, we identified a zinc finger protein Zfp-57 as a self-renewal-specific gene in ES cells by DNA chip analysis, and showed that the expression of Zfp-57 is restricted to undifferentiated ES cells and is regulated by STAT3 and Oct-3/4. Furthermore, we found that Zfp-57 is dispensable for the self-renewal of ES cells.

Our data indicate that Zfp-57 is a downstream target of LIF and STAT3 in ES cells. Recently, Zfp-57 was identified as a novel zinc finger protein of the Schwann cell lineage in mouse and rat sciatic nerves [12]. Interestingly, Zfp-57 mRNA was up-regulated in response to LIF and down-regulated in the presence of a JAK/STAT inhibitor AG490 in Schwann cells [12]. These findings suggested that Zfp-57 is a downstream molecule of the LIF/STAT3 pathway in both ES and Schwann cells.

Our results showed that Zfp-57 expression is regulated by STAT3 and Oct-3/4. Since previous studies showed that both molecules play crucial roles in self-renewal of ES cells [5,6,9,10], it was possible that Zfp-57 could be involved in the self-renewal. However, we were able to establish Zfp-57$^{-/-}$ ES cells, which form compact colonies and express self-renewal markers, including Oct-3/4 and Nanog. These results indicate that Zfp-57 is dispensable for the self-renewal of ES cells. Zfp-57 displays homology in the zinc finger motif with Sp1, Zfp-35, and Rex-1 [11], and in the KARB domain with Zfp-30 [25]. Therefore, it is possible that functional

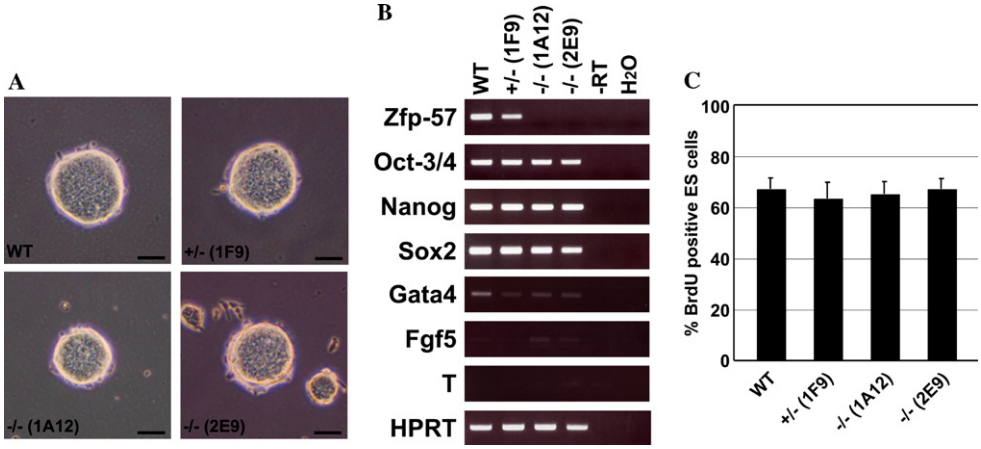

Fig. 6. Zfp-57 is dispensable for the self-renewal of ES cells. (A) Morphology of wild-type (WT), Zfp-57$^{+/-}$ (1F9), and Zfp-57$^{-/-}$ (1A12 and 2E9) ES cells. Scale bars indicate 50 μm. (B) Expression of various marker genes. Total RNAs were isolated from ES cells and subjected to RT-PCR analysis using specific primers for Zfp-57, Oct-3/4, Nanog, Sox2, Gata4, Fgf5, Brachyury T, and HPRT. (C) Proliferation of Zfp-57$^{-/-}$ ES cells. ES cells were subjected to BrdU incorporation assay. The proportion of BrdU-positive cells to PI-positive cells is shown as the mean ± SD for three independent cultures.

redundancy between Zfp-57 and these molecules might compensate for loss of Zfp-57 in ES cells.

Zfp-57 is known to suppress the activity of myelin basic protein promoter in Schwann cells and to localize to the nucleus with a characteristic speckled pattern in NIH3T3 cells [12]. Notably, Zfp-57 co-localizes with heterochromatin protein 1a in Schwann cells. These findings indicate that Zfp-57 functions as a transcriptional repressor, although direct target genes of Zfp-57 have yet to be elucidated in ES and Schwann cells.

Induction of ES cell differentiation by forming embryoid bodies (EBs) in suspension culture provides an in vitro system for studying early lineage determination during mouse development [26]. We thus examined the differentiation capacity of Zfp-57$^{-/-}$ ES cells with this system (data not shown). Expression patterns of differentiation marker genes—such as Fgf5 (embryonic ectoderm), Gata4 (primitive endoderm), and Brachyury T (mesoderm)—were normal in Zfp-57$^{-/-}$ EBs when compared with wild-type EBs, suggesting that Zfp-57$^{-/-}$ ES cells can differentiate into the three germ layers, at least in this assay.

Our present data indicate that Zfp-57 is dispensable for the self-renewal of ES cells and probably for early lineage determination. In embryonic and adult tissues, Zfp-57 expresses in testis, cerebellum, and neuronal tissues, including oligodendrocytes and Schwann cells [11,12]. These observations raise the possibility that Zfp-57 might function in late embryogenesis or neonatal development such as spermatogenesis or myelination. Further analysis, including generation of Zfp-57-deficient mice and identification of a downstream molecule of Zfp-57 by the use of Zfp-57$^{-/-}$ ES cells, will contribute to the understanding of the function of Zfp-57 protein.

Acknowledgments

We are grateful to Drs. Shinwa Shibata, Miwako Kajihara-Miura, and other members of our laboratory for their helpful discussions. We also thank the Center for Biomedical Research and Education at Kanazawa University for the use of the DNA sequencer. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan.

References

[1] M.J. Evans, M.H. Kaufman, Establishment in culture of pluripotential cells from mouse embryos, Nature 292 (1981) 154–156.
[2] G.R. Martin, Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells, Proc. Natl. Acad. Sci. USA 78 (1981) 7634–7638.
[3] A.G. Smith, J.K. Heath, D.D. Donaldson, G.G. Wong, J. Moreau, M. Stahl, D. Rogers, Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides, Nature 336 (1988) 688–690.
[4] R.L. Williams, D.J. Hilton, S. Pease, T.A. Willson, C.L. Stewart, D.P. Gearing, E.F. Wagner, D. Metcalf, N.A. Nicola, N.M. Gough, Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells, Nature 336 (1988) 684–687.
[5] T. Matsuda, T. Nakamura, K. Nakao, T. Arai, M. Katsuki, T. Heike, T. Yokota, STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells, EMBO J. 18 (1999) 4261–4269.
[6] H. Niwa, T. Burdon, I. Chambers, A. Smith, Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3, Genes Dev. 12 (1998) 2048–2060.
[7] K. Okamoto, H. Okazawa, A. Okuda, M. Sakai, M. Muramatsu, H. Hamada, A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells, Cell 60 (1990) 461–472.
[8] H.R. Scholer, G.R. Dressler, R. Balling, H. Rohdewohld, P. Gruss, Oct-4: a germline-specific transcription factor mapping to the mouse t-complex, EMBO J. 9 (1990) 2185–2195.
[9] J. Nichols, B. Zevnik, K. Anastassiadis, H. Niwa, D. Klewe-Nebenius, I. Chambers, H. Scholer, A. Smith, Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4, Cell 95 (1998) 379–391.
[10] H. Niwa, J. Miyazaki, A.G. Smith, Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells, Nat. Genet. 24 (2000) 372–376.
[11] S. Okazaki, S. Tanase, B.K. Choudhury, K. Setoyama, R. Miura, M. Ogawa, C. Setoyama, A novel nuclear protein with zinc fingers down-regulated during early mammalian cell differentiation, J. Biol. Chem. 269 (1994) 6900–6907.
[12] M.B. Alonso, G. Zoidl, C. Taveggia, F. Bosse, C. Zoidl, M. Rahman, E. Parmantier, C.H. Dean, B.S. Harris, L. Wrabetz, H.W. Muller, K.R. Jessen, R. Mirsky, Identification and characterization of ZFP-57, a novel zinc finger transcription factor in the mammalian peripheral nervous system, J. Biol. Chem. 279 (2004) 25653–25664.
[13] S. Azuma, Y. Toyoda, Production of a germ-line chimeric mouse derived from newly established embryonic stem cells, Jpn. J. Anim. Reprod. 37 (1991) 37–43.
[14] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.
[15] U. Yoshida-Koide, T. Matsuda, K. Saikawa, Y. Nakanuma, T. Yokota, M. Asashima, H. Koide, Involvement of Ras in extraembryonic endoderm differentiation of embryonic stem cells, Biochem. Biophys. Res. Commun. 313 (2004) 475–481.
[16] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
[17] K. Mitsui, Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda, S. Yamanaka, The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells, Cell 113 (2003) 631–642.
[18] P. Mountford, B. Zevnik, A. Duwel, J. Nichols, M. Li, C. Dani, M. Robertson, I. Chambers, A. Smith, Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression, Proc. Natl. Acad. Sci. USA 91 (1994) 4303–4307.
[19] P.W. Laird, A. Zijderveld, K. Linders, M.A. Rudnicki, R. Jaenisch, A. Berns, Simplified mammalian DNA isolation procedure, Nucleic Acids Res. 19 (1991) 4293.
[20] T.S. Tanaka, T. Kunath, W.L. Kimber, S.A. Jaradat, C.A. Stagg, M. Usuda, T. Yokota, H. Niwa, J. Rossant, M.S. Ko, Gene expression profiling of embryo-derived stem cells reveals candidate genes associated with pluripotency and lineage specificity, Genome Res. 12 (2002) 1921–1928.

[21] M. Minami, M. Inoue, S. Wei, K. Takeda, M. Matsumoto, T. Kishimoto, S. Akira, STAT3 activation is a critical step in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line, Proc. Natl. Acad. Sci. USA 93 (1996) 3963–3966.

[22] I. Chambers, D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie, A. Smith, Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells, Cell 113 (2003) 643–655.

[23] A.A. Avilion, S.K. Nicolis, L.H. Pevny, L. Perez, N. Vivian, R. Lovell-Badge, Multipotent cell lineages in early mouse development depend on SOX2 function, Genes Dev. 17 (2003) 126–140.

[24] B.A. Hosler, G.J. LaRosa, J.F. Grippo, L.J. Gudas, Expression of REX-1, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells, Mol. Cell. Biol. 9 (1989) 5623–5629.

[25] P. Denny, A. Ashworth, Zfp30, a KRAB domain containing zinc finger protein gene, maps to mouse chromosome 7, Mamm. Genome 5 (1994) 643–645.

[26] A. Leahy, J.W. Xiong, F. Kuhnert, H. Stuhlmann, Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation, J. Exp. Zool. 284 (1999) 67–81.